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Biomarkers and metal content in white seabream (*Diplodus sargus*) and its relationship with the occurrence of the Abnormal Tough Syndrome

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ABSTRACT

There is still controversy on the cause of the incidence of abnormal tough syndrome (ATS) in some fish species, mainly the white seabream (*Diplodus sargus*). This work analyses the incidence of the ATS in white seabream in areas with a different extent of heavy metal pollution and its possible relationship with fish health. Muscular cholinesterase (ChE) activities were selected to assess their potential as biomarkers of ATS occurrence. Among the trace elements Zn, Cu, Pb, and As in muscle and Cu in liver were significantly higher in the fish from the most polluted marina location. ATS incidence (33 %) was also recorded in this site with individuals showing a significantly lower body condition but ChE activities did not reflect any neurotoxic impact on the fish, neither did detoxification and oxidative stress damage. However, a significant negative association was seen between AChE activity and muscle body burden of Hg, Cu, and Se in fish from the less impacted sites, which suggests a certain neurotoxic modulation by these metals. It also alerts for the consumption of fish from recreational activities that do not follow any regulatory measures.

1. Introduction

For several years, specimens of the white seabream species, *Diplodus sargus*, have been reported to exhibit a muscular alteration upon cooking, commonly described as Abnormal Tough Syndrome (ATS) [14]. The ATS phenomenon has been observed in this species not only in the Mediterranean but also in the European Atlantic and Macaronesia regions [14]. Occasional development of ATS have also been documented in other fish species [29,30]. ATS occurs in certain individuals without any apparent symptoms but which make them unpalatably tough upon cooking. Among the hypothesized causes for this syndrome in white seabream, several authors have ascribed it to the uptake of lipophilic secondary metabolites of an invasive alga, *Caulerpa cylindracea* Sonder (Bryopsidales, Chlorophyta) [20,25,26,35,72,81]. In particular, Felline et al. [25] suggested that the concentration of caulerpin in fish tissues could serve as an indicator of trophic exposure to the invasive alga and might be correlated with observed cellular and physiological alterations but no ATS occurrence was reported. These sub-lethal effects included the inhibition of some enzymes such as acetylcholinesterase (AChE) and acyl CoA oxidase. However, the occurrence of ATS in Atlantic areas from the northwestern coast of Spain and the western coast of France, where no *Caulerpa* incidence has been reported [51], suggests that caulerpin is not the unique underlying cause of this syndrome [14].

Some other hypotheses suggest that ATS occurrence in white seabream could be also due to heavy metals present in their environment or diet. In a recent study, Casadevall et al. [14] detected exceptionally high levels of Cu in the liver of a unique specimen affected by ATS, alongside elevated levels of Hg and other potentially harmful elements, surpassing any levels previously described in the literature [13,54,73]. In addition to being harmful to fish, heavy metals are known to also induce oxidative stress, thereby affecting the quality of their flesh [28,47,74].

A former study by Merciai et al. [54] reported elevated levels of Cu and Zn in white seabream muscle from the Gulf of Roses, suggesting a possible influence of anti-fouling paints used on vessels and mariculture cages. Cu is recognized as a neurotoxic compound capable of inhibiting the activity of cholinesterases (ChEs) [18,19,55,56,64]. Moreover, in

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many aquatic organisms, ChEs are inhibited by Cu both *in vivo* and *in vitro* [15,2,33,63,78,79]. Padrilah et al. [58] found that copper sulfate, even at its lowest concentration (0.2 mg/L), inhibited ChE activity by up to 58 %, with nearly 100 % inhibition observed at the highest concentration tested (20 mg/L). Besides Cu, other metals such as Hg, Cd, and Zn, have been shown to be neurotoxic by inhibiting AChE activity, at least *in vitro* [31]. In addition, metals can induce damage in organisms by elevating internal levels of reactive oxygen species (ROS), leading to oxidative stress and causing lipid peroxidation (LPO), DNA damage, and ultimately, cell death [8].

Given the aforementioned possibilities of either invasive toxic algae and/or metal exposure may affect ChEs in white seabream, the muscular activities corresponding to AChE (EC 3.1.1.7), butyrylcholinesterase (BChE, EC 3.1.1.8), and propionylcholinesterase (PrChE, EC 3.1.1.9) were considered for its potential relationship with ATS occurrence. While the physiological role of AChE is well-established (breaking acetylcholine in neuromuscular junctions), the functions of pseudocholinesterases (BChE and PrChE) and other B-esterases such as carboxylesterases (CEs; EC 3.1.1.1) are less understood due to the absence of recognised natural substrates. However, they appear to be involved in protection, cell regeneration, lipid metabolism, neurogenesis, and neural development [53,83]. Furthermore, their binding affinity to certain chemical classes, sometimes even more so than AChE, supports their protective role and warrants their inclusion in pollution monitoring studies with fish [34,40,70,71].

When cholinesterases are inhibited, there is an accumulation of the transmitter acetylcholine (ACh) at the nerve synapse, disrupting normal nervous system function [32]. These inhibitory effects can have physiological consequences such as altered behavior and reduced motility due to their role in the central nervous system and neuromuscular junctions [56]. Stress conditions, due to either chemical exposure, unfavorable conditions, or fishing handling can prompt anaerobic shifts towards anaerobic energy generation, reflected by increased lactate dehydrogenase (LDH) activity. Emerging evidences broaden the spectrum of chemical classes that can influence the aforementioned enzymatic activities in fish. These include antifoulants [50], petrogenic chemicals [80], polychlorinated biphenyls [11], metals [43], pesticides [3,5] and personal care products such as triclosan [6] and detergents [27,38].

The white seabream, *Diplodus sargus* (Linnaeus, 1758), belongs to the omnivorous Sparidae family and is prevalent in the Mediterranean Sea and the eastern Atlantic Ocean, including the archipelagos of Madeira, Cape Verde, and the Canary Islands [82]. This demersal fish thrives in rocky infralittoral and circalittoral habitats at depths ranging from 0 to 50 m, where it often dominates fish assemblages. Individuals are typically medium-sized with a relatively long lifespan, and maintain an omnivorous benthic diet [65]. Due to its high market value, particularly in local fish markets, the white seabream is targeted by artisanal and recreational fisheries.

This study aimed to assess whether the enzymatic activity of ChEs in the muscle of white seabream sampled in areas exhibiting different degrees of metal pollution could serve as biomarker of ATS occurrence. Considering the former findings by Casadevall et al. [12] highlighting elevated levels of Cu and Hg in liver of an ATS affected specimen, analysis of heavy metals was also included in muscle and liver. Furthermore, a characterisation of ChEs in white seabream was conducted for the first time in this species to validate the analytical protocols. Complementary biomarkers (activities of CE, LDH, and LPO levels) were tested in affected and non-affected individuals by the ATS condition to provide further insight into the underlying mechanisms of this syndrome.

2. Material and methods

2.1. Sample collection

Specimens of white seabream were collected from three ports along the northern Catalan coast (NE Spain): Roses (n = 20), Palamós (n = 21) and Badalona (n = 18) (Fig. 1). The first two sites are regular fishing grounds situated in the open sea, while the third location is a marina situated near a fishing port, a nautical port, and the large metropolitan area of Barcelona. Fish were captured by local artisanal fisheries between 2016 and 2020, and those from Badalona were collected by sport divers in 2021 next to the marina. In winter, this species typically accumulates fat in preparation for gonadal maturation, which is when it typically exhibits its optimal physical condition. By taking the samples in December, we ensured that the condition of these fish was at its best.

Fig. 1. Map of the three sampling locations along the NW Mediterranean Catalan coast where the white seabream (*Diplodus sargus*) were sampled.

All fish were weighted, measured to the nearest centimetre, and visually examined for anomalies. Subsequently, the specimens were dissected to examine the presence of perivisceral and retroperitoneal fat, and the liver was extracted. Muscle samples were obtained by cutting above the lateral line at the highest part of the body. Once all tissue samples were collected for analysis, the remaining part of the fish was baked for 15–20 min, depending on fish size, and the meat was tested to detect ATS occurrence.

2.2. Muscle and liver preparation for biochemical and chemical analysis

For cholinesterase characterisation and other enzymatic determinations, approximately 0.3 g of muscle from the same dorsal site of the fish was dissected and promptly frozen at −20 ºC. Muscle extracts for biochemical determinations were made by homogenising in ice-cold potassium phosphate buffer (50 mM, pH 7.4) containing 1 mM of ethylenediaminetetraacetic acid (EDTA) at a ratio of 1:4 (w:v) using a Polytron® homogeniser. The homogenates were then centrifuged at 10,000 g for 30 min at 4ºC. The resulting supernatant (S10) was aliquoted and stored at −80 ºC for further biochemical analyses.

For chemical analysis, portions of muscle tissue weighing between 150 and 250 mg wet weight (w.w.) and 400 mg w.w. of liver were stored frozen at −20ºC. Chemical analysis in muscle included: Zn, Mn, Pb, Cd, Cu, Sn, Hg, Se, As, and Cr, and in the liver we considered concentration of Cu and Hg, for comparison with previous results from this same species.

2.3. Enzymatic determinations in muscle

For ChE measurements in the muscle S10 fraction, the substrates acetylthiocholine iodide (ATC), butyrylthiocholine iodide (BTC), and propionylthiocholine iodide (PTC) were used following the adapted protocol of Ellman et al. [23] to microplate conditions. A volume of 25 µL sample was adequately diluted (to ensure kinetic linearity) and incubated with DTNB (5, 5′ -dithio-bis- 2-nitrobenzoate) for 2 min to eliminate nonspecific hydrolysis. Following this, 50 µL of each substrate

(1 mM final concentration) was added, and the reaction was monitored for 5 min at 412 nm. Additional determinations (CE, LDH, and LPO) were conducted only in fish from Badalona since some of them (6 out of 18) exhibited ATS.

For CE activity determinations, the substrate p-nitrophenyl butyrate (pNPB) was adopted. In each well, 25 μL of S10 and pNPB at a final concentration of 1 mM were loaded into the microplate well [37]. The sample dilution was also adjusted to ensure kinetic linearity at 405 nm within the 5-minute timeframe measurement.

Lactate dehydrogenase (LDH) activity was assessed followed adaptation of the Vassault [76] method, using a 25 μL (1/200) diluted sample, and NADH and pyruvate at a final concentration of 200 μM and 1 mM, respectively. The reaction was followed for 5 min at 340 nm.

Malondialdehyde content was measured as a LPO indicator following an adaptation of the protocol by Esterbauer et al. [24] using the chromogenic reagent 1-methyl-2-phenylindole. A calibration curve was prepared using 1,1,3,3-tetramethoxypropane standards, and absorbance was read at 586 nm.

All reactions were conducted in triplicate at 25ºC using the kinetic mode (Magellan v6.0) on a TECAN Infinite 200 microplate reader.

2.4. Protein content determination

The protein content of samples was determined using the [9] method adapted to microplate using the Bio-Rad Protein Assay reagent. Additionally, a standard curve was generate using bovine serum albumin (0.05–0.5 mg/ml) to quantify the protein content. Reading was performed at 495 nm, and the total protein content was used to normalize former hydrolysis rates.

2.5. B-esterases characterisation and in vitro inhibition by model and emerging contaminants

In order to validate B-esterases (ChE and CE) measurements, a former *in vitro* characterisation was made, for the first time in this species, using a six-point concentration range (0.3–10 mM) of the three ChE substrates. Kinetic parameters such as Vmax, Km, and catalytic efficiency were calculated using the Generalized Reduced Gradient (GRG) nonlinear algorithm. Additionally, further *in vitro* characterisation of ChEs was made with the aid of model chemicals used as specific inhibitors: BW284c51 (BW; CAS 402–40-4) for AChE determinations, eserine (CAS 57–47-6) for all ChEs, and tetraisopropyl pyrophosphoramide (ISO-OMPA; CAS 513–00-8) as specific for BuChE measurements. CE enzymes were identified by the use of bis(4-nitrophenyl) phosphate (BNPP; CAS 645–15-8). Additionally, we included other chemicals of environmental concern that had revealed to act as CE inhibitors: the flame retardants tetrabrombisphenol A (TBBPA; CAS 79–94-7) and the TCPP isomer mixture (TCPP, CAS 13674–48-5).

In vitro tests were conducted with pooled fish muscle homogenates (n = 4). The chemicals were diluted in potassium phosphate buffer (50 mM pH 7.4) and ethanol:buffer solution at a 1:1 (v/v) ratio. Before this inhibitory test, it was verified that the proportion of ethanol used in the incubation did not affect the enzymatic measurements. A single concentration of 100 μM in the incubation mixture was used for all the selected chemicals. The muscle S10 fraction was incubated with 10 μL of each chemical or carrier at room temperature with gentle shaking for 15 min. Subsequently, the substrates were added to 25 μL of the pre-incubated sample, and the residual activity was measured at 412 nm (for ChEs) or 405 nm (for CE) during 5 min, following the procedure described in Section 2.3.

2.6. Chemical analysis of heavy metals in muscle and liver

Muscle and liver samples were digested in Teflon vessels using 2 ml of HNO_3 and 1 ml of H_2O_2 (Merck, Suprapure) at 90 °C in an oven overnight. All materials involved in the digestion process were meticulously acid-rinsed. After digestion, samples were diluted with 30 ml of Milli-Q water. Total concentrations of trace elements were quantified by inductively coupled plasma-mass spectrometry (ICP-MS, Perkin Elmer Elan 6000). Mercury analyzed encompassed total Hg, including inorganic Hg (iHg), MeHg, and any other mercury forms present. The analytical procedure was checked using standard reference materials: Dogfish (*Squalus acanthias*) liver (DOLT-3) and muscle (DORM-2) from the National Research Council of Canada.

Several analytical blanks were prepared and analyzed alongside samples to establish detection limits, which were consistently below 0.1 ng ml^{-1} for all elements. Recovery rates consistently ranged from 90 to 110 %. The analytical process was conducted at the CCiTUB (Centres Científics i Tecnològics de la Universitat de Barcelona).

2.7. Statistical analysis

The length-weight relationship was established using linear regression analysis, comparing eviscerated weight (EW) to total length (TL) after logarithmic transformation: EW = log(a) + b log (TL), where “a” represents the intercept of the regression curve (a coefficient associated with body form), and “b” is the regression coefficient. Deviation of “b” from the “ideal value” of 3, which signifies isometric growth [62], indicates changes in body proportions as fish length increases. When “b” is less than 3, fish become slimmer with increasing length, indicating negatively allometric growth.

Le Cren’s relative condition factor (Kn) was calculated as follows: Kn = EW/a TLb, where “a” and “b” are constants. Additionally, Fulton’s condition Factor (K), was calculated using the formula: K = EW/TL3 × 100. This morphometric index suggests that heavier fish of a given length are in better condition, serving as a straightforward indicator of energy storage.

Normality and homoscedasticity were assessed using the Shapiro-Wilks and Levene tests, respectively. Data were transformed in order to meet these assumptions when necessary. A t-Student test was conducted to compare metal levels in the muscle and liver of healthy animals (n = 12) versus affected ones (n = 6) from the location of Badalona. A Spearman correlation test was performed to assess the relationship between B-esterases activities and bioaccumulated metals in the muscle of fish. To detect significant differences (p-value < 0.05) in muscle heavy metal loads between areas, Kruskal-Wallis and post-hoc Dunn tests with Bonferroni correction were employed. Additionally, these tests explored differences in Cu and Hg levels in the liver among different sites.

Table 1
Number of individuals of white seabream (*Diplodus sargus*) analyzed, condition factors, and biometrics from the fish from each location. Data for the Badalona area are presented collectively and separately for individuals with or without abnormal tough syndrome (ATS). TL = total length, EW = eviscerated weight, Kn = Le Cren's condition index, K = Fulton’s condition index.

	N	TL (cm)	EW (g)	Kn	K	Total length/weight relationship	R^2
Roses	20	23.5–31.3	249–727	1.03	1.93	y = 2.8826x – 10.604	0.91
Palamós	21	25.5–34.5	272–794	1.03	1.88	y = 3.2153x – 12.114	0.91
Badalona (all specimens)	18	19.5–32.5	119–454	0.99	1.65	y = 2.5484x – 8.5087	0.86
• No ATS	12	19.5–32.0	171–454	1.00	1.70	y = 2.4114x – 2.1627	0.76
• ATS	6	21.5–30.0	119–441	1.00	1.56	y = 2.5782x – 2.8041	0.98

3. Results

3.1. Biometric and enzymatic activities in white seabream

Table 1 presents the biometric measurements and condition indices for specimens collected from the three locations, along with the number of individuals with ATS at each site. The gross morphometric condition index highlighted that specimens from the Badalona marina exhibited a poorer physical condition, which corresponded to the only site where ATS individuals were found (33 %). Conversely, white seabream from Roses and Palamós exhibited similar body condition values with a Le Cren condition factor close to one, and the regression factor approached the "ideal value" of three. However, fish from Badalona fell below the reference value, irrespective of ATS occurrence. The condition factor, K, further emphasised that specimens from Roses and Palamós were heavier for a given length compared to those from Badalona. It is noteworthy that despite being collected during the optimal winter period (December) for fat reserves, specimens from Badalona lacked the perivisceral and retroperitoneal fat, typically observed in this species during this period.

Muscle ChE activities (in nmol/min/mg protein) ranged as follows: AChE from 9.3 to 56.7, BuChE from 7.7 to 32.9 and PrChE from 7.3 to 38.0 with mean values per location indicated in Table 2. Moreover, muscle ChE activity decreased with increasing body size ($r = -0.57$; $p < 0.05$). There were no significant differences per site, even after considering fish length in the ANCOVA model, as size as a factor to influence this activity. Additionally, no significant differences were seen between the Badalona specimens exhibiting or not ATS in terms of ChEs or any of the additional parameters analysed (CE and LDH activities or LPO levels) (Table 3).

The characterisation of ChEs confirmed that the substrate concentrations (1 mM) were adequate for the respective measurements in this fish species, revealing a Michelis-Menten trend of values for Vmax, Km, and catalytic efficiency (Vmax/Km) (Table 4). Additionally, the true nature of the measurements was confirmed by employing specific inhibitors tested at single 100 μM concentration. AChE was inhibited by BW 284c51 (65.4 ± 2.4 %), BuChE by iso-OMPA (95.7 ± 1.1 %), and PrChE by eserine (98.0 ± 0.4 %).

CE measurements using the substrate pNPB also revealed a significant inhibition by the organophosphorous pesticide BNPP, accounting for 40.7 ± 2.9 % of this activity in the muscle of white seabream. Two additional contaminants of emerging concern, the halogenated flame retardant (TBBPA) and an isomer mixture or organophosphorous flame retardants (TCPP), were examined for any *in vitro* interaction with muscular ChEs and pNPB-CE. Only TBBPA caused a significant inhibition of basal pNPB-CE activity, with a reduction of 22.21 ± 1.4 %, while ChEs were not significantly affected (ranging from 2.8 to 13.9 %). The impact of TCPP was even less significant, with inhibitions ranging from 4.2 to 17.2 %, but not reaching statistical significance (Fig. 2).

3.2. Analysis of metals in muscle and liver of white seabream

Table 5 provides an overview of the concentration of Zn, Mn, Pb, Cd, Cu, Sn, Hg, Se, As, and Cr, in muscle of fish from each location. The Kruskal-Wallis test revealed a significantly higher concentration of all heavy metals and As in muscle in fish from Badalona marina, being specially remarkable for Zn, Pb, Cu, Hg, and As. Conversely, Mn and Cd exhibited a higher concentration in Roses samples compared to Badalona, albeit not significantly.

Table 6 reports on the liver bioaccumulation of Cu and Hg in fish from the three sampling sites. In all cases, levels of Cu in the liver were substantially higher than in muscle. Those of Hg did not show significant variation per site and they were slightly elevated in the liver, except in Badalona, where they preferentially accumulated in muscle. Unexpectedly, the liver from fish from Palamós exhibited the highest value of Hg compared with the other two sites, while those from Badalona displayed the highest Cu load. Fish from Roses showed intermediate values for both toxic metals. Kruskal-Wallis analysis revealed significant differences between Badalona and the other two sites for Cu, whereas, for Hg, significant differences were observed between Palamós and the other two locations.

3.3. Correlation between muscular enzymatic activities, fish size and metal content

In Fig. 3, the correlogram illustrates the relationship between enzyme activities of ChEs, fish size, and bioaccumulated metals in the muscle of white seabream from the three sites. Size was a factor to negatively affect ChE activities ($\rho = 0.44$–0.56; $n = 59$), and the three ChEs exhibited strong positive correlations among themselves ($\rho = 0.92$–0.95; $n = 59$). Other associations were observed between muscular ChEs and Hg content of negative sign ($\rho = 0.30$–0.36; $n = 59$), and Se ($\rho = 0.26$). Consequently, fish size was also related to the bioaccumulation of metals, but in a positive manner, Hg ($\rho = 0.62$), Cu ($\rho = 0.37$), As ($\rho = 0.28$), and Se ($\rho = 0.48$).

4. Discussion

Gross morphometric markers are likely indicative of physiological disturbances previously taking place at lower levels of biological organisation, such as the biochemical ones. Morphometric condition indicators are straightforward reliable measures of energy storage in fish, which serve as a proxy for fitness playing a crucial role on survival and reproductive performance in fish [48]. White seabream is a capital breeder fish, which means that obtains most of the energy required for reproduction and maintenance from lipid reserves in the form of perivisceral fat, gained typically before and during feeding periods [12,39]. Specifically, this species accumulates lipids from November to March [60]. For that, we intentionally captured fish from the most polluted area (Badalona) in December, to ensure that they were in peak optimal body condition in terms of fat reserves. Unexpectedly, fish from this marina were notably thin, with a complete absence of perivisceral and retroperitoneal fat. This precarious health condition became even more evident when comparing them with the fish from the other two locations. Remarkably, in this work, ATS individuals were only confirmed in the Badalona site at a significant proportion (33 %). Formerly, Casadevall et al. [12] reported only one ATS specimen in the Roses area, out of a sample size of 42 individuals, representing therefore, a much smaller proportion. However, it is important to note that flesh hardness is not exclusively related to the low lipid reserves since in this former study, none of the fish lacking lipidic reserves exhibited ATS. In contrast, the single individual developing ATS, had muscle fat content

Table 2
Mean ± SD for biometric length (in cm) and Mean ± SEM for cholinesterase activities (in nmol/min/mg protein) in muscle from white seabream (*Diplodus sargus*) from the three sampling locations.

Location	n	Total length	AChE	BChE	PChE
Roses	20	22.3 ± 4.42	22.98 ± 2.77	14.86 ± 1.50	18.11 ± 1.54
Palamós	21	25.0 ± 3.64	19.12 ± 1.45	12.17 ± 1.11	16.00 ± 0.89
Badalona	18	25.6 ± 3.40	22.14 ± 1.71	13.18 ± 1.10	19.45 ± 1.48

Table 3
Biomarker activities in muscle of white seabream (*Diplodus sargus)* from the most marina location (Badalona), in specimens affected and non-affected by the Abnormal Tough Syndrome (ATS). Activities (as mean ± SEM) of acetylcholinesterase (AChE), butyrylcholinesterase (BChE), propionylcholinesterase (PChE), carboxylesterase with p-nitrophenyl butyrate as substrate (p-NPB-CE), lactate dehydrogenase (LDH) and lipid peroxidation (LPO) levels. Activities in nmol/min/mg prot and LPO levels in nmol MDA/g w.w.

	n	Length	AChE	BChE	PChE	pNPB - CE	LDH	LPO
No ATS	12	25.83 ± 2.88	20.90 ± 7.96	12.36 ± 5.18	18.48 ± 7.17	4.20 ± 1.46	1297.4 ± 283.57	9.14 ± 7.84
ATS	6	25.17 ± 4.62	24.61 ± 5.27	14.82 ± 3.19	21.41 ± 3.65	4.67 ± 0.87	1242.6 ± 471.09	7.51 ± 4.59

Table 4
Kinetic properties of AChE, BChE and PrChE Vmax (in nmol/min/mg protein), Km (in mM) and catalytic efficiency (Vmax/Km) in muscle of white seabream (*Dioplodus sargus*).

Substrate	Vmax	Km	Kcat
ATC	34.70 ± 3.71	0.44 ± 0.07	81.62 ± 8.80
BTC	25.59 ± 2.88	0.15 ± 0.01	171.97 ± 11.97
PTC	49.32 ± 5.27	0.30 ± 0.02	163.75 ± 10.99

above the average and one of the highest perivisceral and retroperitoneal fat contents. In this survey, fish from the marina of Badalona had a precarious health condition and zero fat reserves, but as discussed above, this does not appear to be the only factor for developing ATS.

In the search to identify any underlying muscular biochemical parameters that could relate to the fish physiological condition causing or associated with ATS occurrence, we considered biomarkers of neurotoxicity, xenobiotic metabolism, and oxidative stress damage. The rational for choosing ChEs, as a potential biomarker of ATS occurrence, is provided by the two formerly mentioned hypothesis: 1) ingestion of the invasive *Caulerpa* spp. and 2) metal exposure, as both can cause neurotoxicity. No occurrence of *Caulerpa* spp. was detected at the fishing sites but metal exposure notably differed per location. Additionally, hydrolytic pNPB-CE activity and lipid peroxidation levels were also contrasted in the muscle tissue of fish affected and unaffected by ATS.

Our results did not show an association between ATS development and muscle enzymatic activities, since ChEs and the other selected

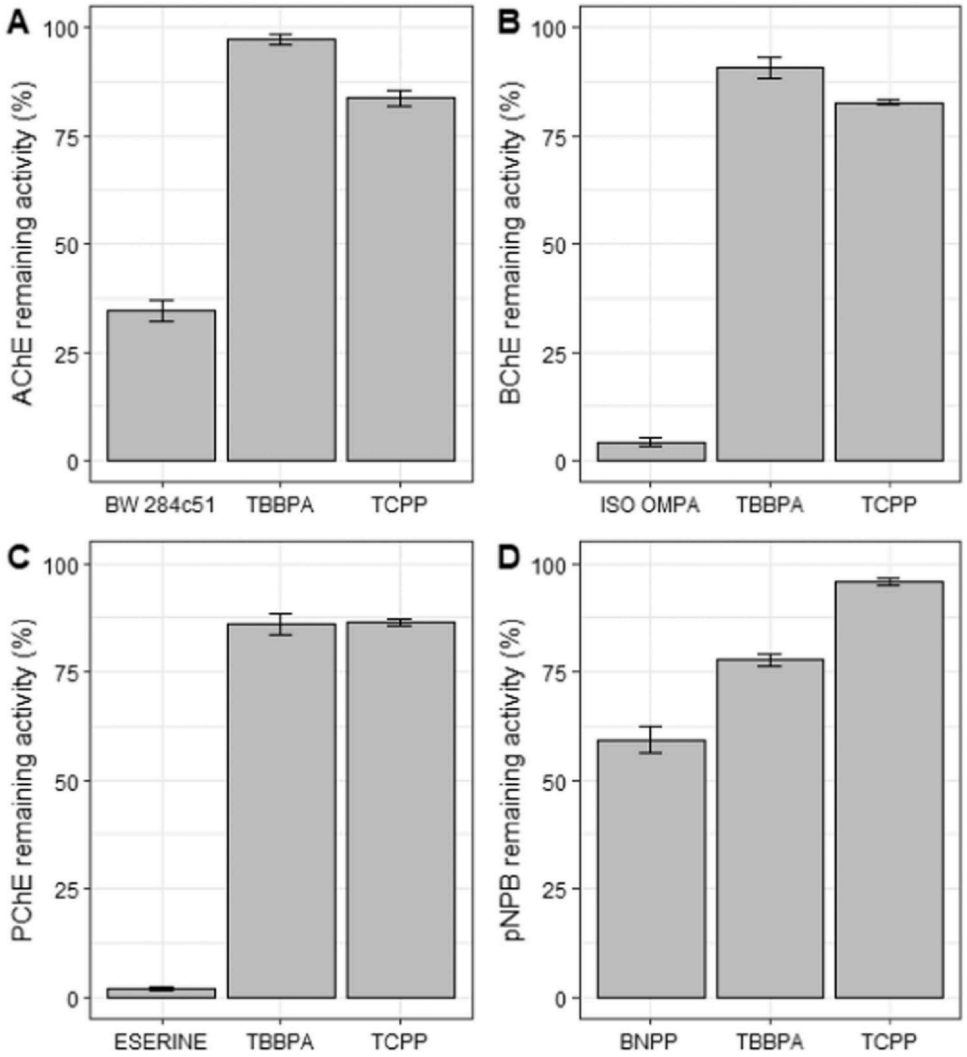

Fig. 2. Percentage of residual cholinesterases (AChE, BChE, PChE) and carboxylesterase (pNPB-CE) activities in muscle S10 fraction of white seabream (*Diplodus sargus*), in respect to controls after 15 min incubation with the model inhibitor and contaminants of emerging concern. Data are mean ± SEM (n = 3). Model chemicals BW284c51, eserine and ISO-OMPA; Tetrabromobisphenol A (TBBPA, CAS 79-94-7) and the TCPP isomer mixture (TCPP, CAS 13674-48-5).

Table 5
Data summary of white seabream (*Diplodus sargus)* samples, trace-element concentrations in the muscle for the three sample areas (mean ± SD). All elements concentrations are expressed in μg/kg (wet weight). Kruskal-Wallis and post-hoc Dunn test are represented with capital letter in brackets indicate significant differences (p-value < 0.05) between areas.

Metal	Roses (n = 20)	Palamós (n = 21)	Badalona (n = 18)
Zn	4,225.3 ± 261.6 (A)	4,121.3 ± 283.6 (A)	16,349.1 ± 1167.8 (B)
Mn	614.4 ± 168.9 (AB)	339.5 ± 161.0 (A)	323.8 ± 26.4 (B)
Pb	26.8 ± 7.2 (A)	19.2 ± 5.1 (A)	213.4 ± 67.7 (B)
Cd	4.6 ± 0.8 (A)	1.2 ± 0.2 (B)	2.3 ± 0.3 (AB)
Cu	183.3 ± 14.5 (A)	228.7 ± 21.8 (A)	877.7 ± 126.0 (B)
Sn	7.3 ± 2.1 (A)	7.6 ± 1.4 (A)	28.8 ± 4.3 (B)
Hg	299.0 ± 65.1 (A)	426.0 ± 86. 9 (A)	741.3 ± 74.8 (B)
Se	295.8 ± 15.8 (A)	361.5 ± 27.9 (A)	886.4 ± 47.7 (B)
As	7,492.8 ± 1351.6 (A)	4,479.4 ± 591.7 (A)	19,412.9 ± 3110.5 (B)
Cr	70.5 ± 10.7 (A)	344.0 ± 172.9 (A)	372.4 ± 63.8 (B)

Table 6
Data (mean ± SD) summary of white seabream (*Diplodus sargus)* samples, trace-element concentrations in the liver for the three sampling areas. All elements concentrations in μg/kg w.w.(wet weight).

Metal	Roses (n = 20)	Palamós (n = 22)	Badalona (n = 18)
Cu	12,043.6 ± 19,023.2 (B)	29,339.2 ± 28,079.9 (B)	78,884.4 ± 39,303.4 (A)
Hg	365.7 ± 461.2 (A)	1,349.8 ± 993.1 (B)	555.6 ± 259.4 (A)

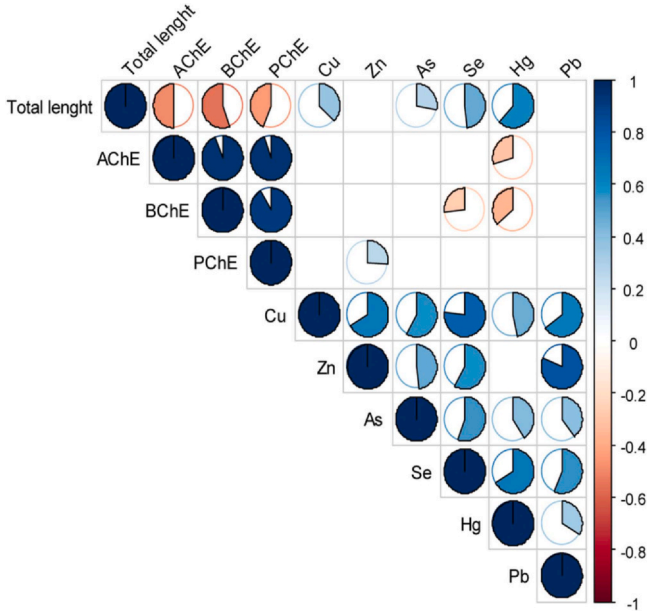

Fig. 3. Spearman correlations between total length, enzyme activities of ChEs (AChE, BChE and PChE) and bioaccumulated metals in muscle of white seabream (*Diplodus sargus)* from the three sampling sites. Only significant correlations (p-value < 0.05; n = 59) are indicated.

biomarkers were comparable across the three sampling locations, as well as between specimens developing ATS or not. To the best of our knowledge, ChE activities have not previously been reported in white seabream but the mean values attained for AChE (20.16 nmol/min/mg protein) in the 3 sampling sites was lower than activity (58.4–120.5 nmol/min/mg protein) for other fish of the same Sparidae family from a nearby area [67]. Muscular ChEs hydrolysis rates with the assayed substrates showed a preference (ATC > PTC > BTC), that slightly differed in *Dicentrarchus labrax* (ATC > BTC > PTC), although they are all of a similar magnitude [75]. In another related species, *Sparus aurata*, muscular AChE and BChE displayed hydrolysis rates in the range of white seabream and although the substrate preference was age-dependent, they were also of a similar magnitude [5]. The kinetic parameters defining ChEs in white seabream are similar to those described in *S. aurata* by Albendín et al. [4], confirming the adequacy of the 1 mM substrate concentration for the measurements in both sparids. The use of specific inhibitors in the present study with white seabream further confirmed the true nature of the measurements and the inclusion of two flame retardants revealed similar responsiveness to chemicals of environmental concern, as reported in other marine species [57,68,69].

The results of the present study also confirmed the general observation that muscle ChE decreases with increasing body size as formerly revealed in *S. aurata,* particularly for AChE more so than BChE [5]. Therefore, size was considered a cofactor when assessing site differences or the effect of ChE responses or metals because the effect of size may potentially mask the results. Our observations also confirmed a positive size-related trend in Hg accumulation over time, which is already well-known in fish muscle tissue. This trend in white seabream was also recently highlighted by Casadevall et al. [13] and Merciai et al. [54]. Hg values were higher in muscle, but it would only be a matter of concern for consumers of Badalona fish (0.7 ppm), which surpassed the limit of 0.5 ppm stablished by the EU legislation [21]. Related to fish, Hg both in its organic and inorganic form, was reported to have a negative impact on white seabream motility [59,61]. The level of Hg in liver was also considered for its implication in fish health. In this case, it was the fish from Palamós the ones that showed the highest levels in liver, although in muscle was lower than those reported from fish from the Badalona marina. This trend indicates that, despite being exposed to high levels of this naturally occurring pollutant in Palamós [41], the detoxification activity of the liver is efficient. The good condition in fish from Palamós likely allows them to successfully face the greater energy demands that enhanced detoxification processes require [49].

Another metal of concern for its potential action on ChEs is Cu. Little information is available on liver loads of this heavy metal in white seabream, but the few data available indicates that reported values are in mean lower than the ones revealed in the present study. Kress et al. [42] found levels of around 7 ± 2 mg/kg of Cu in white seabream liver from the eastern Mediterranean area and Afonso et al. [1] of 23.66 ± 10.25 mg/kg in fish from the Atlantic coast of the Canary Islands. In the presently surveyed area, Casadevall et al. [12] detected relevant Cu concentrations in the liver of white seabream from the fishing area of Cap de Creus, between 8 and 125 mg/kg, with exceptionally high values of Cu in the liver of two individuals: 748 and 837 mg/kg, corresponding this last value to an individual developing ATS. Leaving aside the two extreme values, our present results (between 3.2 and 187.8 mg/kg) are quite similar, even though those found in Badalona were among the highest (between 31.43 mg/kg and

187.8 mg/kg). This is not surprising due to the presence of a nearby fishing port and intense nautical activities that use anti-fouling paints (based on Cu) in vessels [36,44]. In aquaculture, it is known that high concentrations and prolonged exposure to Cu can result in the mortality of white seabream [77]. Consistent with our results, Vaz et al. [77] observed that the liver of this fish species exhibited a higher concentration of Cu than muscle tissue. The mechanisms of action for Cu toxicity include disruption of ion regulation, induction of oxidative stress, effects on liver metabolism, impacts on bioenergetics leading to impaired growth and reproduction, and effects on sensory systems [10]. Moreover, Cu accumulation rates may surpass detoxification rates, leading to hepatic oxidative stress [52], and increased liver detoxification may incur higher bioenergetic costs [10]. It has been demonstrated in several species that the muscle is not an active tissue for heavy metal accumulation (except for Hg), and this only occurs when the maximum storage capacity of the liver is exceeded [45,84]. Other metals such as Zn and Pb, and As were also notably high in fish muscle from Badalona. Zn (likewise Cu) may originate from anti-fouling paints [36,44], while As contamination is believed to stem from herbicides, pesticides, and fungicides [16,46]. Lead is mainly related to Pb additives in gasoline [17]. The fact that the Badalona specimens were captured next to a large metropolitan area may be the reason of high Pb levels in this fish samples. This arises concern for consumers since the European Food Safety Authority [22] recommends a PTWI (Provisional Tolerable Weekly Intake) of 25 μg/kg for Pb, and fish from Badalona show an almost 10-fold higher content (mean of 213.36 ± 67.69 μg/kg?.

Overall, in this study, a clear relationship between ChE activities and the other contrasted biomarkers (CE and LPO), metal accumulation, and ATS occurrence could not be established, mainly due to the high metal overload in fish from the marina site. However, a significant negative association seen between AChE activity and muscle body burden of Hg, Cu, and Se in fish from the less polluted sites, suggests a certain enzymatic modulation by metals, where Se presence may have a buffering role of preventing Hg toxicity [66]. Our results did not prove that metals presence was responsible for the modulation of ChE activities, which may indicate this species uses other buffering strategies in metal protection. We would like also to highlight the importance of monitoring levels of toxic metals in marinas, as many recreational fisheries take place nearby, and fish consumption from these areas is not yet regulated. In particular, Cu concentrations in marinas can rise swiftly following the introduction of boats, potentially reaching toxic levels. Measures such as controlling the number of boats or reducing the use of copper-based paints [7] could help to reduce these levels and their unwanted consequences for wildlife and human health.

5. Conclusions

Despite a significantly lower body condition and higher trace elements levels in muscle from white seabream from the Badalona marina location, where ATS incidence was also recorded, ChE activities did not reflect any neurotoxic impact on the fish. However, a significant negative association was seen between AChE activity and muscle body burden of Hg, Cu, and Se in fish from the less polluted sites, suggesting a certain enzymatic modulation by metals. In fish liver, levels of Hg and Cu did not apparently have an impact on fish health; however, it is worth noting that fish across Catalan coast could be affected by the factors responsible for ATS at different extent, even if they did not show any apparent symptoms. In this study, the occurrence of ATS could not be monitored by muscular ChE activities or by any other biomarker indicative of hydrolytic activity or oxidative stress. Thus, ChE values recorded in white seabream from the NW Mediterranean coast likely reflect baseline levels. Further studies are needed to unravel the mechanisms responsible for ATS occurrence and its monitoring.

CRediT authorship contribution statement

Margarida Casadevall: Conceptualization, Data curation, Investigation, Resources, Writing – original draft, Writing – review & editing. **Montserrat Solé:** Formal analysis, Investigation, Supervision, Writing – original draft, Writing – review & editing. **Sergi Omedes:** Data curation, Formal analysis, Writing – review & editing. **Conxi Rodríguez-Prieto:** Conceptualization, Investigation, Writing – review & editing. **María Lorenzo:** Formal analysis, Methodology, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Montserrat Sole reports equipment, drugs, or supplies was provided by Spanish Scientific Research Council. The corresponding author is in the editorial board of EPM. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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